QuantiGene® Plex 2.0 Reagent System

User Manual

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Panomics

Panomics, Inc.

QuantiGene Plex 2.0 Reagent System User Manual

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Section I: Introduction

About This User Manual

Who Should Read This Manual

This manual is for anyone who has purchased QuantiGene Plex 2.0 Assay Kits from Panomics to perform the QuantiGene Plex assay for any of the following sample types:

- Cultured cells
- Blood samples (whole blood, PAXgene blood, dried blood spots)
- Fresh, frozen, or formalin-fixed, paraffin-embedded (FFPE) animal tissues
- Purified RNA

What This Manual Covers

This manual provides recommendations and step-by-step procedures for the following:

- Experimental design and data analysis
- QuantiGene Plex 2.0 assay
- Troubleshooting

Safety Warnings and Precautions

All chemicals should be considered potentially hazardous. We recommend that this product and its components be handled by those trained in laboratory techniques and used according to the principles of good laboratory practice.

For research use only. Not for use in diagnosis of disease in humans or animals.

Contacting Panomics

Technical Help

For technical questions, please contact our technical support group by telephone at 1-877-726-6642 option 3, or email at techsupport@panomics.com (US and Canada). In Europe, contact techsupport_europe@panomics.com. In Asia Pacific, contact techsupport_asia@panomics.com. For an updated list of FAQs and product support literature, visit our website www.panomics.com.

About the QuantiGene Plex 2.0 Reagent System

Introduction

The QuantiGene Plex 2.0 Reagent System is designed to quantitate, in a single well, multiple target-specific RNA molecules present in:

- Cultured cell lysates
- Whole blood, PAXgene blood RNA, or dried blood spot lysates
- Fresh or frozen animal tissue homogenates
- FFPE animal tissue homogenates
- Total RNA, mRNA, or *in vitro* transcribed RNA preparations

Please refer to Panomics' QuantiGene Sample Processing Kit Product Inserts for instructions on preparing cultured cell or whole blood lysates or animal tissue homogenates. To prepare RNA, follow standard laboratory methods.

QuantiGene Plex 2.0 Assay Basics

Assay Technology

The QuantiGene Plex 2.0 assay combines branched DNA (bDNA) signal amplification and multi-analyte profiling beads (xMAP®) technologies to enable the detection and quantitation of multiple mRNA targets simultaneously.

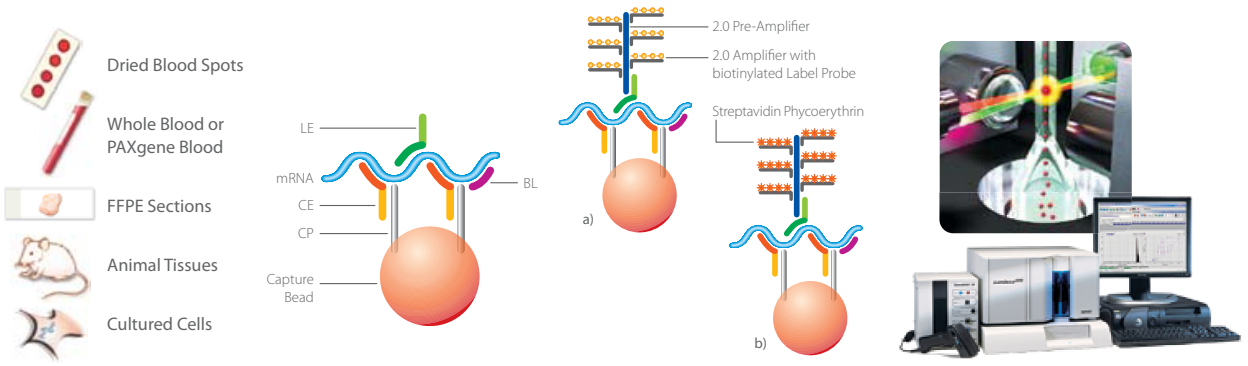
The bDNA assay is a hybridization-based method of target-specific RNA quantitation that amplifies signal rather than target RNA, using labeled DNA probes.

The xMAP system combines a flow cytometer, fluorescent-dyed microspheres (beads), lasers and digital signal processing to allow multiplexing of up to 100 unique assays within a single sample. The QuantiGene Plex assay is compatible with all Luminex-based instruments currently available.

Assay Principal

The QuantiGene Plex 2.0 system utilizes fluorescent microspheres (Capture Beads) as a support to capture specific RNA molecules. The ability to quantify multiple target-specific RNA molecules in a single sample lies in the design of the Probe Sets. For each RNA molecule of interest, an oligonucleotide Probe Set containing three types of synthetic probes, Capture Extenders (CEs), Label Extenders (LEs), and Blockers (BLs) that hybridize and span contiguous sequences of the target RNA, is provided. The CEs discriminate among the different Capture Beads within the bead array while capturing, via cooperative hybridization, the target RNA.

Signal amplification is mediated by DNA amplification molecules that hybridize to the tails of the LEs. Each amplification unit contains multiple hybridization sites for biotinylated Label Probes that bind Streptavidin-conjugated R-Phycoerythrin (SAPE). The resulting fluorescence signal associated with individual Capture Beads is read on a Luminex flow cytometer. Signal is reported as median fluorescence intensity (MFI) and is proportional to the number of target RNA molecules present in the sample.



Step 1: Release Target RNA

Cells are lysed to release RNA.

Step 2: Target RNA Capture

Specific mRNA transcripts are captured to their respective beads through a Capture Extender (CE) Capture Probe (CP) interaction during an overnight hybridization at 54°C.

Step 3: Signal Amplification

a) Sequential hybridization of the 2.0 Pre-Amplifier, 2.0 Amplifier and biotinylated Label Probe, respectively, for an hour at 50°C.

b) Binding with Streptavidin-conjugated Phycoerythrin (SAPE) at room temperature for 30 minutes.

Step 4: Detection

The sample is analyzed on a Luminex* instrument. The level of SAPE fluorescence is proportional to the amount of mRNA transcripts captured by the bead.

** Bio-Plex suspension array system or other Luminex-based array systems.*

QuantiGene Plex 2.0 Reagent System Contents and Storage Conditions

QuantiGene Plex Assay Kit

The components of the QuantiGene Plex 2.0 Assay Kit and their recommended storage conditions are listed below.The QuantiGene Plex Assay Kit is available in 3 sizes. Refer to the product insert for quantities of individual components supplied. Kit components have a shelf life of 6 months from the date of receipt.

Each Assay Kit contains the following components:

Component	Description	Storage
Proteinase K[a]	Proteinase K in aqueous buffered solution	–20 °C
Blocking Reagent	Aqueous buffered solution containing a preservative	–20 °C
Label Probe	Biotinylated oligonucleotide in aqueous buffered solution	–20 °C
2.0 Pre-Amplifier	DNA in aqueous buffered solution	–20 °C
2.0 Amplifier	DNA in aqueous buffered solution	–20 °C
Amplifier Diluent	Aqueous buffered solution containing protein and a preservative	2–8 °C
Label Probe Diluent	Aqueous buffered solution containing protein and a preservative	2–8 °C
SAPE	Streptavidin-conjugated R-Phycoerythrin	2–8 °C
SAPE Diluent	Aqueous buffered solution containing protein and a preservative	2–8 °C
Lysis Mixture	Aqueous buffered solution containing a preservative	15–30 °C
2.0 Wash Buffer Component 1	Aqueous solution	15–30 °C

Each Assay Kit contains the following components: *(continued)*

Component	Description	Storage
2.0 Wash Buffer Component 2	Aqueous buffered solution	15–30 °C
SAPE Wash Buffer	Aqueous buffered solution	15–30 °C
Filter Plate	96-well filter plate	15–30 °C
Filter Plate Holder	96-well, clear bottom polystyrene microplate	15–30 °C
Plate Seals	Adhesive backed foil plate sealer	15–30 °C
Hybridization Plate Assembly (1- and 3-plate Assay Kits)	96-well, clear polypropylene plate, sealing mat, and plate lid	15–30 °C
Plate Clamps (1- and 3-plate Assay Kits)	Larger binder clips for securing Hybridization Plate Assembly	15–30 °C
Heat-Sealable Hybridization Plate (10-plate Assay Kit)	96-well, clear polypropylene plate	15–30 °C
Heat-Sealing Foil (10-plate Assay Kit)	Heat-sealing foil plate seal	15–30 °C

a. We recommend storing in an enzyme storage box, such as the NEB Cool Box (New England Biolabs P/N T0400S). NEVER store at –80 °C.

Accessory Reagents

In addition to QuantiGene Plex 2.0 Assay Kits, two accessory reagents are required to perform QuantiGene 2.0 Plex assays.

For ordering information, please visit our website at www.panomics.com

Accessory Reagent	Description/Comment
QuantiGene Sample Processing Kit	Contains reagents and instructions for processing different sample types. Specify sample type (cultured cells, blood samples, FFPE samples, fresh or frozen animal tissue).
QuantiGene Plex 2.0, Plex Sets	Contains pre-pooled target-specific 2.0 Probe Set and pre-pooled 2.0 Capture Beads or 2.0 Mag Capture Beads.

Required Reagents and Equipment

About Required Materials

These materials are required to perform the assay but are not supplied with the QuantiGene Plex Assay Kit.

Equipment and Disposables

Item	Source
Adjustable single- and multi-channel precision pipettes for dispensing 1–20 µL, 20–200 µL and 200–1,000 µL	Major Laboratory Supplier (MLS)

Item	Source
Reagent reservoirs: 25 mL capacity, divided 25 mL capacity 100 mL capacity	VistaLab Technologies (P/N 3054-1004 or equivalent) (P/N 3054-1002 or equivalent) Corning Costar (P/N CLS 4873 or equivalent)
Microcentrifuge	Eppendorf 5415D or equivalent
Microplate centrifuge that can achieve 240 x g	Eppendorf 5804R and rotor A-2-DWP or equivalent
Vortex mixer	MLS
RNase-free water	MLS
Microcentrifuge tubes	MLS
Vacuum filtration system or ELx50 Microplate Strip Washer with filter plate option	Millipore (P/N MAVM0960R and WP6111560) BioTek ELx50/8FMW
Shaking incubator with microplate adaptor, capable of maintaining constant temperatures of 50 °C ± 1 °C and 54 °C ± 1 °C, speed of 600 rpm, and 3 mm orbit	LabNet VorTemp 56 (P/N S-2056-Q)[a]
Microplate shaker, capable of maintaining a speed of 600 rpm, 0.12 inch orbit	Labline model 4625 or equivalent
Heat block at 95 °C	MLS
Luminex or Luminex-based instrument	MiraiBio, Bio-Rad or other Luminex instrument provider
Thermo-seal heat sealer (for 10-plate Assay Kits)	ABGene (P/N AB-0384 and AB-0563/1000) or equivalent
QuantiGene Incubator Temperature Validation Kit	Panomics (P/N QS0517)

a. If an alternative is used, see “Appendix IV: Validating Alternative Shaking Incubators” on page 37 for guidelines on validating a non-Vortemp shaking oven.

Guidelines for Assay Design and Analysis

Overview

Here we provide information and guidelines on the following:

- Optimizing sample input
- Determining complete cell lysis
- Replicate recommendations
- Assay background controls
- Use of housekeeping genes
- Data analysis

Optimizing Sample Input

The sensitivity of the QuantiGene Plex 2.0 assay is approximately 5,000 RNA copies per target per well when using 2.0 Capture Beads (polystyrene) and 1,500 RNA copies per target per well when using 2.0 Mag Capture Beads (magnetic).

When running a sample type for the first time, and/or using a new target-specific Plex Set, we strongly recommend that you optimize sample input to ensure the signals are within the assay and instrument linear range. Luminex instruments exhibit saturation at approximately 20,000 MFI. In our experience, assay linear working ranges are approximately 5K–5,000K RNA transcripts when using 2.0 Capture Beads and 1.5K–1,500K RNA transcripts when using 2.0 Mag Capture Beads.

We provide recommendations for typical starting sample inputs for each sample type, see “Capturing Target RNA” on page 15. Using these recommendations as a guide, perform a 2-fold dilution series of your sample and verify that the resulting assay signals are linearly proportional to sample input. For more information, see “Determining Assay Linearity” on page 12.

Determining Complete Cell Lysis

Optimal QuantiGene Plex assay performance depends on the complete release and stabilization of the RNA from the cells and protein complexes.

Incomplete cell lysis may result in:

- Poor assay precision
- High CV values
- Non-linear assay

If any of these conditions occur, your samples may not be completely lysed.

Complete cell lysis depends on:

- Correct ratio of cells to lysis solution (Working Lysis Mixture or Working Homogenization Solution)
- Method used to lyse the cells

Guidelines on cell/lysis solution ratios and lysis methods can be found in the QuantiGene Sample Processing Kit Product Inserts. We strongly recommend running an optimization experiment in which different amounts of samples are used with a constant amount of appropriate lysis solution to ensure assay linearity and precision.

Determining the Correct Cell/Lysis Solution Ratio

For example, if you are lysing cell cultures, prepare lysate at 400 cells/µL. To optimize this, make additional lysates at 200 and 800 cells/µL. Make serial dilutions of each lysate and evaluate for viscosity, assay CV, and assay linearity.

Optimizing the Lysis Method

Start with the recommended procedure to get optimal results. Subsequently, you can modify the procedure as needed to simplify the process. Regardless of the process used, evaluate the assay linearity and precision to ensure optimal and consistent results.

Replicate Recommendations

Technical replicates are replicate assays from a single sample. For example, a cell lysate that is divided into several portions and each portion run in the same QuantiGene Plex 2.0 assay.

Biological replicates are replicate assays from biologically-equivalent samples. For example, cells grown in different wells that are subjected to the same treatment, lysed independently, then run as distinct samples in the QuantiGene Plex 2.0 assay.

We recommend running 3 technical replicates of each distinct biological sample.

Recommended Assay Controls

Assay Background Control

Assay background is the signal (median fluorescence intensity, MFI) generated by the assay components in the absence input RNA.

Run an assay background control, in triplicate, for each Plex Set used in each experiment.

Use of Housekeeping Genes

A *Housekeeping gene* is a target gene that is stably expressed under all experimental conditions evaluated. An important aspect of a housekeeping gene is its relative abundance. Ideally, housekeeping genes expression levels should be similar to the target genes in your Plex Set so that a single sample input results in a linear response for all targets. See “Appendix III: Common Housekeeping Genes and their Relative Expression Levels” on page 36 for a list of common housekeeping genes and their relative abundance. Signals for housekeeping genes can be used to normalize gene expression data across samples. Include one or more housekeeping genes in the design of every Plex Set.

Data Analysis Guidelines

Calculating Assay Precision

The Coefficient of Variation (CV) is a measure of assay precision. QuantiGene Plex 2.0 Assay CVs are typically less than 15% for technical replicates.

To determine the assay CV:

Step	Action
1	Run technical replicates of each sample.
2	Calculate the average signal (AVG) of technical replicates for each RNA.
3	Calculate the standard deviation (SD) of signals from technical replicates for each RNA.
4	Calculate the %CV. %CV = (SD/AVG)*100.

Calculating Assay Limit of Detection

Calculate assay limit of detection (LOD) for each RNA as follows:

LOD = AVG RLU of assay background control wells + 3X SD of assay background signals.

Assay signals below LOD should not be used to draw quantitative conclusions about gene expression.

Determining Assay Linearity

To determine the assay linearity:

Step	Action
1	Run a dilution series of your sample.
2	Subtract the AVG assay background signal from the AVG signal of technical replicates for each RNA.
3	Calculate the ratio of background-subtracted AVG RLU from sequential sample dilutions for each RNA. Observed values should be within 20% of the expected ratio of 100% (80%–120%). For example, % of Obs/Exp = Obs/Exp x 100 where Obs = Observed background subtract average RLU and Exp = Expected fold change based on dilution. **3-fold serial dilution of the cell lysate (µL) / Signal (background subtracted) (MFI) / Observed fold change / Expected fold change / % Obs/Exp** 60 / 3100 / 3.10 / 3 / 103 20 / 1000 / 2.70 / 3 / 90 6.6 / 370 / / / Quantifiable signals are those signals within the assay linear range.

Normalizing Gene Expression Data

To normalize gene expression data:

Step	Action
1	Subtract the AVG assay background signal from the AVG signal of technical replicates for each RNA.
2	Divide the background-subtracted, AVG signals for each target RNA by the background-subtracted, AVG signal of the housekeeping RNA. **Note** If multiple housekeeping RNAs are measured, the geometric mean of background-subtracted AVG housekeeping RNA signals may be used for data normalization.

Calculating Fold-Change

To calculate fold-change of an RNA target in treated versus untreated samples:

Step	Action
1	Normalize gene expression data as described above.
2	Divide the normalized value for the treated sample by the normalized value for the untreated sample.

Section II: QuantiGene Plex 2.0 Assay Procedure

About the Assay Procedure

Overview A general outline of the assay procedure is as follows:

Sample Preparation

Refer to the appropriate QuantiGene Sample Processing Kit Product Insert for sample type-specific instructions for preparing cultured cell and whole blood lysates or tissue homogenates. Follow standard laboratory methods for purification of RNA.

Use samples immediately in QuantiGene Plex 2.0 assays, or store at –80 °C until use.

Instrument and Equipment Setup

- Setup filtration system
- Setup Luminex instrument
- Validate Luminex instrument setup and operation

Target RNA Capture

- Dilute samples, if appropriate
- Prepare Working Plex Sets
- Dispense Working Plex Sets, samples and controls into Hybridization Plates
- Hybridize samples overnight

Signal Amplification and Detection of Target RNA

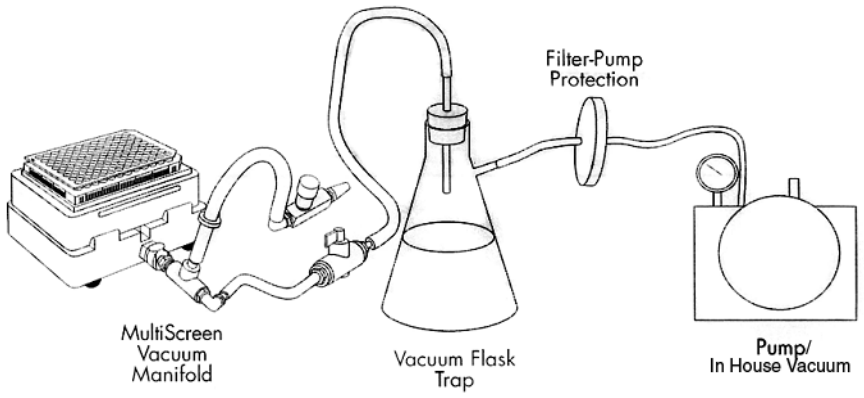
- Transfer samples to Filter Plate, and filter away unbound material
- Sequentially hybridize the 2.0 Pre-Amplifier, 2.0 Amplifier, Label Probe and SAPE
- Analyze samples using a Luminex-based instrument

Instrument and Equipment Setup

Filtration System Setup and Operation

Refer to the manufacturer's recommended protocols to configure the filter plate vacuum manifold as shown below and adjust the vacuum to 1–2 inches of Hg.

Note See "Appendix V: Automated Filter Plate Washing Method (optional)" on page 39 for alternative, automated filter plate washing method.



To verify the performance of the vacuum manifold:

Step	Action
1	Transfer a Filter Plate containing 100 µL of 2.0 Wash Buffer/well to the vacuum manifold and firmly press down on all four corners of the plate to ensure a good seal. **Note** Refer to "Prepare 2.0 Wash Buffer" on page 25 for instructions on preparing 2.0 Wash Buffer.
2	Turn on the vacuum.
3	Completely filter the solution from all wells.
4	Turn off the vacuum. Filtration rate should be 5–15 seconds. If necessary, adjust the pressure. **IMPORTANT** Do not let the FIlter Plate dry completely. Following filtration, the bottom of the Filter Plate should look translucent and not white. Filtering too long or at too high a pressure can result in significant Capture Bead loss.

Luminex Setup and Operation

Follow the manufacturer's recommended protocol for general operation and maintenance for your instrument. See "Appendix II: Recommended Maintenance for Luminex Instruments" on page 35 for information on cleaning maintenance of your instrument.

The QuantiGene Plex 2.0 assay is optimized for use at the low sensitivity setting. The sensitivity setting is performed during calibration. Refer to the table in "Appendix I: Setting Up Luminex Instrument for QuantiGene Plex 2.0 Assays" on page 34 for information on setting up your Luminex instrument.

IMPORTANT Refer to the QuaniGene Plex 2.0 Plex Set Product Insert for the target—bead region associations for your panel.

Validating Luminex Instrument Calibration and Setup

IMPORTANT Perform this procedure before you run the assay, to ensure the Luminex instrument is setup correctly for your QuantiGene Plex assay.

To verify the instrument calibration and setup:

Step	Action
1	**Note** Setup the Luminex instrument according to the guidelines in “Appendix I: Setting Up Luminex Instrument for QuantiGene Plex 2.0 Assays” on page 34.
2	Define a protocol with the appropriate bead regions and set to read 2 wells. **IMPORTANT** Refer to the QuantiGene Plate 2.0 Plex Set Product Insert for the target—bead associations for your panel.
3	Pre-wet the Filter Plate by adding 100 µL SAPE Wash Buffer to 2 wells, then filter.
4	Vortex 2.0 Capture Beads or 2.0 Mag Capture Beads at maximum speed for 30 seconds.
5	Add 1 µL of 2.0 Capture Beads or 2.0 Mag Capture Beads to 300 µL of SAPE Wash Buffer. Vortex to mix.
6	Add 130 µL of the Capture Bead mixture into each of the 2 pre-wet wells in the Filter Plate.
7	Insert the Filter Plate into the instrument and read the 2 wells.
8	View the window with the bead regions and DD gate. The expected results are: ♦ Signals for the expected beads show up on the bead map ♦ Average bead count is greater than 50/region ♦ Single peak in the DD gate window with signals within the set DD gate region.

Capturing Target RNA

About Capturing Target RNA

This section provides three protocols for capturing target RNA based on sample type:

a. Cultured cell and whole blood lysates
b. Fresh, frozen, or FFPE animal tissue homogenates
c. Total RNA, mRNA, or *in vitro* transcribed RNA preparations

Refer to the appropriate protocol for your sample type.

Capturing Target RNA from Cultured Cell and Whole Blood Lysates

Procedure

To capture target RNA from cultured cell and whole blood lysates:

Step	Action
1	Prepare the following reagents: ♦ Probe Set and Blocking Reagent. Thaw, vortex briefly to mix, then centrifuge briefly to collect contents at the bottom of the tube. ♦ Lysis Mixture. Re-dissolve any precipitates by incubating at 37 °C for 30 minutes followed by gentle swirling. ♦ Capture Beads. Take out of storage right before use and protect from light. ♦ Proteinase K. Take out of storage right before use and place on ice. ♦ Cultured cell or whole blood lysates. If frozen, thaw at room temperature followed by incubation at 37 °C for 30 minutes. For tubes, vortex briefly, for plates, pipette up and down 5 times, then leave at room temperature until use. **IMPORTANT** Do not put samples back on ice.
2	Transfer an appropriate volume (5 µL/assay well, plus 40% overage) of Probe Set to a microfuge tube, heat to 95 °C for 5 minutes using a heat block, then place on ice.
3	Pre-warm Lysis Mixture at 37 °C for 30 minutes followed by gentle swirling.
4	If appropriate, based on the expression level of lowest abundance target or housekeeping RNA in the Plex Set, **dilute samples with Diluted Lysis Mixture** (1 volume of Lysis Mixture plus 2 volumes nuclease-free water, prepared fresh) so that the desired amount of sample is present in a volume of 80 µL/assay well. Use the table below as a guide, and scale dilution volumes according to the number of assays to be run. **IMPORTANT** Always verify that assay signals are within both the instrument and assay linear ranges. For more information, see “Optimizing Sample Input” on page 9, “Determining Complete Cell Lysis” on page 10, and “Determining Assay Linearity” on page 12.

Recommended Sample Input			
	Cultured Cells (number of cells)		
RNA (copies per cell)	2.0 Capture Beads	2.0 Mag Capture Beads	Blood Lysate (µL)
1	32,000	20,000	80[a]
10	5,000	2,000	80
100	500	200	8
≥1000	≤ 50	≤ 20	≤ 0.08

a. May not have the sensitivity required.

To capture target RNA from cultured cell and whole blood lysates: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>5</td><td>Prepare an appropriate volume of Working Plex Set by combining the following reagents in the order listed. Scale according to the number of assays to be run, and include 40% overage. Use the table below as a guide.

IMPORTANT Vortex (at maximal speed) Capture Beads for 30 seconds before addition.

<table>
<tr><th>Order of Addition</th><th>Reagent</th><th>1 Well (µL)</th><th>48 Wells[a] (µL)</th><th>96 Wells[a] (µL)</th></tr>
<tr><td>1</td><td>RNase-free water</td><td>5.2</td><td>349</td><td>697</td></tr>
<tr><td>2</td><td>Lysis Mixture</td><td>6.6</td><td>442</td><td>884</td></tr>
<tr><td>3</td><td>Blocking Reagent</td><td>2</td><td>134</td><td>268</td></tr>
<tr><td>4</td><td>Proteinase K</td><td>0.2</td><td>13</td><td>27</td></tr>
<tr><td>5</td><td>2.0 Capture Beads</td><td>1</td><td>67</td><td>134</td></tr>
<tr><td>6</td><td>2.0 Probe Set</td><td>5</td><td>335</td><td>670</td></tr>
<tr><td colspan="2">Total</td><td>20</td><td>1,340</td><td>2,680</td></tr>
</table>
a. Includes 40% overage.</td></tr>
<tr><td>6</td><td>Vortex Working Plex Set for 30 seconds to mix, then dispense into the Hybridization Plate.

For fewer than 48 wells:

Using a single channel pipette and a new tip for each transfer, dispense 20 µL Working Plex Set into each well of the Hybridization Plate.

For 48 wells or more:
a. Using a single channel pipette, transfer Working Plex Set to a 25-mL capacity-divided reagent reservoir.

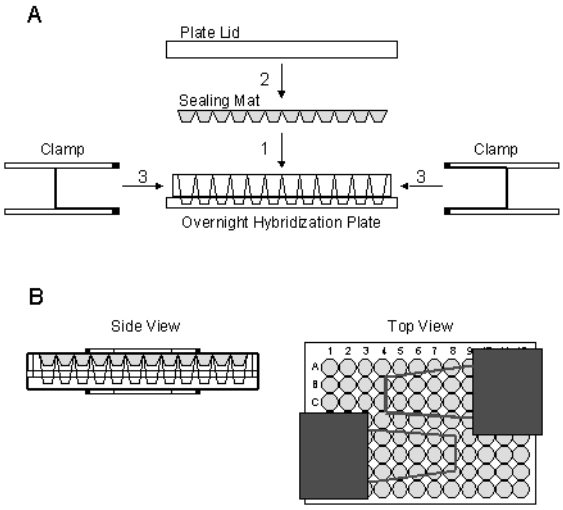
Note Do not pour or reagent shortage will occur.

b. Using a multichannel pipette and new tips for each transfer, dispense 20 µL of Working Plex Set into each well of the Hybridization Plate.

IMPORTANT Include 3 wells for assay background control.</td></tr>
<tr><td>7</td><td>Using a new pipette tip for each transfer, add 80 µL cell lysate or diluted cell lysate to each well of the Hybridization Plate containing Working Plex Set.

IMPORTANT Add 80 µL of Diluted Lysis Mixture (1 volume of Lysis Mixture plus 2 volumes of nuclease-free water) to 3 wells for assay background control.</td></tr>
</table>

To capture target RNA from cultured cell and whole blood lysates: *(continued)*

Step	Action
8	Use one of the following methods to seal the plate: ♦ **If using components from a 1- or 3-plate Assay Kit**: a. Tightly seal the Hybridization Plate using the Sealing Mat. **IMPORTANT** Ensure that each individual well of the sealing mat has been snapped into place. Failure to completely seal the plate may result in excessive evaporation during the overnight incubation. b. Cover the plate with the plate lid and secure it with a clamp at each end as shown below. **Note** If clamped plates do not fit in the incubator holder, heavy-duty rubber bands may be substituted for the clamps.  ♦ **If using components from a 10-plate Assay Kit, seal the Hybridization Plate(s) with Heat-Sealing Foil using the ABgene heat sealer**: a. Center the seal on the Hybridization Plate with white side facing up and silver side contacting the surface of the plate. b. Place the Hybridization Plate squarely onto the accessory plate carrier. c. Press down firmly to seal for 5 full seconds. d. Turn plate 180 degrees and repeat step c.
9	Place an inverted Plate Lid into each of the two plate carriers in the Vortemp shaking Incubator, then place the Hybridization Plate on top of the inverted Plate Lid. If running a single plate, place another filter plate with a plate seal in the second position for balance. Incubate for 18–22 hours at **54 °C ± 1 °C** and 600 rpm. **IMPORTANT** Use a QuantiGene Incubator Temperature Validation Kit to verify the assay temperature. In the case of Vortemp instruments with the inverted plate lids in place, the Vortemp digital display and the actual temperature measured by the QuantiGene Incubator Temperature Validation Kit thermocouple (inserted into the mock Hybridization Plate) may differ by 4 °C or more.

Capturing Target RNA from Fresh, Frozen, or FFPE Animal Tissue Homogenates

Procedure

To capture target RNA from tissue homogenates:

Step	Action
1	Prepare the following reagents: ♦ Probe Set and Blocking Reagent. Thaw, vortex briefly to mix, then centrifuge briefly to collect contents at the bottom of the tube. ♦ Lysis Mixture. Re-dissolve any precipitates by incubating at 37 °C for 30 minutes followed by gentle swirling. ♦ Capture Beads. Take out of storage right before use and protect from light. ♦ Proteinase K. Take out of storage right before use and place on ice. ♦ Tissue homogenates. If frozen, thaw at room temperature followed by incubation at 37 °C for 30 minutes. For tubes, vortex briefly, for plates, pipet up and down 5 times, then keep at room temperature until use. **IMPORTANT** Do not put samples back on ice.
2	Transfer an appropriate volume (5 µL/assay well, plus 40% overage) of Probe Set to a microfuge tube, heat to 95 °C for 5 minutes using a heat block, then place on ice.
3	Pre-warm Lysis Mixture at 37 °C for 30 minutes followed by gentle swirling.
4	If appropriate, **dilute tissue homogenates with Homogenizing Solution** so that the desired amount of sample is present in a volume of 40 µL/assay well based on the expression level of lowest abundance target or housekeeping RNA in the Plex Set. Use the table below as a guide, and scale dilution volumes according to the number of assays to be run. **IMPORTANT** Always verify that assay signals are within both the instrument and assay linear ranges. For more information, see “Optimizing Sample Input” on page 9, “Determining Complete Cell Lysis” on page 10, and “Determining Assay Linearity” on page 12.

Recommended Sample Input	
RNA (copies per cell)	**Tissue Homogenates (µL)**
1	40[a]
10	40
100	4
≥ 1,000	≤ 0.4

a. May not have sensitivity required.

To capture target RNA from tissue homogenates: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>5</td><td>Prepare an appropriate volume of Working Plex Set by combining the following reagents, in the order listed. Scale according to the number of assays to be run, and include 40% overage.

IMPORTANT Vortex (at maximal speed) Capture Beads for 30 seconds before addition.

<table>
<tr><th>Order of Addition</th><th>Reagent</th><th>1 Well (µL)</th><th>48 Wells[a] (µL)</th><th>96 Wells[a] (µL)</th></tr>
<tr><td>1</td><td>RNase-free water</td><td>18.5</td><td>1,240</td><td>2,479</td></tr>
<tr><td>2</td><td>Lysis Mixture</td><td>33.3</td><td>2,231</td><td>4,462</td></tr>
<tr><td>3</td><td>Blocking Reagent</td><td>2</td><td>134</td><td>268</td></tr>
<tr><td>4</td><td>Proteinase K</td><td>0.2</td><td>13</td><td>27</td></tr>
<tr><td>5</td><td>2.0 Capture Beads</td><td>1</td><td>67</td><td>134</td></tr>
<tr><td>6</td><td>2.0 Probe Set</td><td>5</td><td>335</td><td>670</td></tr>
<tr><td colspan="2">Total</td><td>60</td><td>4,020</td><td>8,040</td></tr>
</table>
a. Includes 40% overage.</td></tr>
<tr><td>6</td><td>Vortex Working Plex Set for 30 seconds to mix, then dispense into the Hybridization Plate.

For fewer than 48 wells:

Using a single channel pipette and a new tip for each transfer, dispense 60 µL Working Plex Set into each well of the Hybridization Plate.

For 48 wells or more:
a. Using a single channel pipette, transfer Working Plex Set to a 25-mL capacity divided-reagent reservoir.

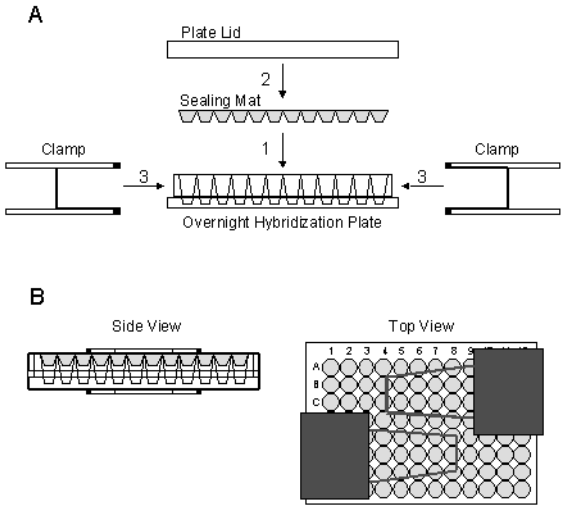
Note Do not pour or reagent shortage will occur.

b. Using a multichannel pipette and new tips for each transfer, dispense 60 µL of Working Plex Set into each well of the Hybridization Plate.

IMPORTANT Include 3 wells for assay background control.</td></tr>
<tr><td>7</td><td>Using a new pipette tip for each transfer, add 40 µL tissue homogenate or diluted tissue homogenate to each well of the Hybridization Plate containing Working Plex Set.

IMPORTANT Add 40 µL of Homogenizing Solution to 3 wells for assay background control.</td></tr>
</table>

To capture target RNA from tissue homogenates: *(continued)*

Step	Action
8	Use one of the following methods to seal the plate: ♦ **If using components from a 1- or 3-plate Assay Kit**: a. Tightly seal the Hybridization Plate using the Sealing Mat. **IMPORTANT** Ensure that each individual well of the sealing mat has been snapped into place. Failure to completely seal the plate may result in excessive evaporation during the overnight incubation. b. Cover the plate with the plate lid and secure it with a clamp at each end as shown below. **Note** If clamped plates do not fit in the incubator holder, heavy-duty rubber bands may be substituted for the clamps.  ♦ **If using components from a 10-plate Assay Kit, seal the Hybridization Plate(s) with Heat-Sealing Foil using the ABgene heat sealer**: a. Center the seal on the Hybridization Plate with white side facing up and silver side contacting the surface of the plate. b. Place the Hybridization Plate squarely onto the accessory plate carrier. c. Press down firmly to seal for 5 full seconds. d. Turn plate 180 degrees and repeat step c.
9	Place an inverted Plate Lid into each of the two plate carriers in the Vortemp shaking Incubator, then place the Hybridization Plate on top of the inverted Plate Lid. If running a single plate, place another filter plate with a plate seal in the second position for balance. Incubate for 18–22 hours at **54 °C ± 1 °C** and 600 rpm. **IMPORTANT** Use a QuantiGene Incubator Temperature Validation Kit to verify the assay temperature. In the case of Vortemp instruments with the inverted plate lids in place, the Vortemp digital display and the actual temperature measured by the QuantiGene Incubator Temperature Validation Kit thermocouple (inserted into the mock Hybridization Plate) may differ by 4 °C or more.

Capturing Target RNA from Total, Purified or *In Vitro* Transcribed RNA

Procedure

To capture target RNA from purified RNA preparations:

Step	Action
1	Prepare the following reagents: ♦ Lysis Mixture. Re-dissolve any precipitates by incubating at 37 °C for 30 minutes followed by gentle swirling. ♦ Probe Set and Blocking Reagent. Thaw, vortex briefly to mix, then centrifuge briefly to collect contents to the bottom of the tube. ♦ Capture Beads. Take out of storage right before use and protect from light. ♦ RNA sample(s). If frozen, thaw on ice. Vortex briefly before use.
2	Transfer an appropriate volume (5 µL/assay well, plus 40% overage) of Probe Set to a microfuge tube, heat to 95 °C for 5 minutes using a heat block, then place on ice.
3	Pre-warm Lysis Mixture at 37 °C for 30 minutes followed by gentle swirling.
4	If appropriate, based on expression level of the lowest abundance target or housekeeping RNA in the Plex Set (for total RNA or mRNA), **dilute RNA in nuclease-free water** so that the desired amount of RNA is present in a volume of 20 µL/assay well. Use the table below as a guide, and scale dilution volumes according to the number of assays to be run. **IMPORTANT** Always verify that assay signals are within both the instrument and assay linear ranges. For more information, see “Optimizing Sample Input” on page 9 and “Determining Assay Linearity” on page 12.

Recommended Sample Input

RNA (copies per cell)	Total RNA		mRNA (ng)	
	2.0 Capture Beads	2.0 Mag Capture Beads	2.0 Capture Beads	2.0 Mag Capture Beads
1	2,500	300	50	6
10	250	30	5	0.6
100	25	3	0.5	0.06
≥1000	≤ 2.5	≤ 0.3	≤ 0.05	≤ 0.006

For *in vitro* transcribed (IVT) RNA, recommended input is ≥ 20,000 copies of each target RNA per well. We recommend including 200 ng/µL yeast tRNA in IVT RNA dilutions to minimize RNA loss.

To capture target RNA from purified RNA preparations: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>5</td><td>Prepare an appropriate volume of Working Plex Set by combining the following reagents in the order listed. Scale according to the number of assays to be run, and include 40% overage. Use the table below as a guide.

IMPORTANT Vortex (at maximum speed) Capture Beads for 30 seconds before addition.

<table>
<tr><th>Order of Addition</th><th>Reagent</th><th>1 Well (µL)</th><th>48 Wells[a] (µL)</th><th>96 Wells[a] (µL)</th></tr>
<tr><td>1</td><td>RNAse-free water</td><td>38.7</td><td>2,593</td><td>5,186</td></tr>
<tr><td>2</td><td>Blocking Reagent</td><td>2</td><td>134</td><td>268</td></tr>
<tr><td>3</td><td>Lysis Mixture</td><td>33.3</td><td>2,231</td><td>4,462</td></tr>
<tr><td>4</td><td>2.0 Capture Beads</td><td>1</td><td>67</td><td>134</td></tr>
<tr><td>5</td><td>2.0 Probe Set</td><td>5</td><td>335</td><td>670</td></tr>
<tr><td colspan="2">Total</td><td>80</td><td>5,360</td><td>10,720</td></tr>
</table>
a. Includes 40% overage.</td></tr>
<tr><td>6</td><td>Vortex Working Plex Set for 30 seconds to mix, then dispense into the Hybridization Plate.

For fewer than 48 wells:

Using a single channel pipette and a new tip for each transfer, dispense 80 µL Working Plex Set into each well of the Hybridization Plate.

For 48 wells or more:

a. Using a single channel pipette, transfer Working Plex Set to a 25-mL capacity divided-reagent reservoir.

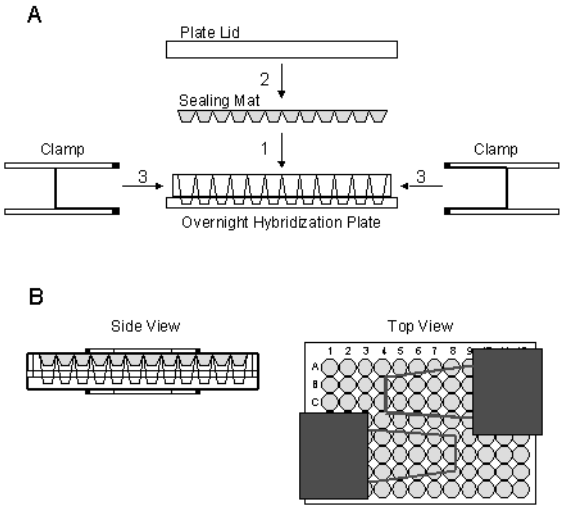
Note Do not pour or reagent shortage will occur.

b. Using a multichannel pipette and new tips for each transfer, dispense 80 µL of Working Plex Set into each well of the Hybridization Plate.

IMPORTANT Include 3 wells for assay background control.</td></tr>
<tr><td>7</td><td>Using a new pipette tip for each transfer, add 20 µL RNA sample to each well of the Hybridization Plate containing the Working Plex Set.

IMPORTANT Add 20 µL RNase-free water to 3 wells for assay background control. For IVT RNA background controls, add 20 µL RNase-free water containing 200 ng/µL yeast tRNA.</td></tr>
</table>

To capture target RNA from purified RNA preparations: *(continued)*

Step	Action
8	Use one of the following methods to seal the plate: ♦ **If using components from a 1- or 3-plate Assay Kit**: a. Tightly seal the Hybridization Plate using the Sealing Mat. **IMPORTANT** Ensure that each individual well of the sealing mat has been snapped into place. Failure to completely seal the plate may result in excessive evaporation during the overnight incubation. b. Cover the plate with the plate lid and secure it with a clamp at each end as shown below. **Note** If clamped plates do not fit in the incubator holder, heavy-duty rubber bands may be substituted for the clamps.  ♦ **If using components from a 10-plate Assay Kit, seal the Hybridization Plate(s) with Heat-Sealing Foil using the ABgene heat sealer**: a. Center the seal on the Hybridization Plate with white side facing up and silver side contacting the surface of the plate. b. Place the Hybridization Plate squarely onto the accessory plate carrier. c. Press down firmly to seal for 5 full seconds. d. Turn plate 180 degrees and repeat step c.
9	Place an inverted Plate Lid into each of the two plate carriers in the Vortemp shaking Incubator, then place the Hybridization Plate on top of the inverted Plate Lid. If running a single plate, place another filter plate with a plate seal in the second position for balance. Incubate for 18–22 hours at **54 °C ± 1 °C** and 600 rpm. **IMPORTANT** Use a QuantiGene Incubator Temperature Validation Kit to verify the assay temperature. In the case of Vortemp instruments with the inverted plate lids in place, the Vortemp digital display and the actual temperature measured by the QuantiGene Incubator Temperature Validation Kit thermocouple (inserted into the mock Hybridization Plate) may differ by 4 °C or more.

Signal Amplification and Detection of RNA Targets

About this Procedure

These instructions are for processing one 96-well plate using multichannel pipettes and reagent reservoirs. To process fewer or more wells, scale reagents accordingly.

Before You Start

Bring Amplifier Diluent, Label Probe Diluent, and SAPE Diluent to room temperature. **Warm Amplifier Diluent to 37 °C for 20 minutes** to dissolve any precipitates, and mix well by inversion before use.

Prepare 2.0 Wash Buffer

To prepare 2.0 Wash Buffer:

Step	Action
1	Add to a 250-mL graduated cylinder, in this order: ♦ 190 mL nuclease-free water ♦ 0.6 mL 2.0 Wash Buffer Component 1 ♦ 10 mL 2.0 Wash Buffer Component 2 **Note** Scale preparation according to the number of plates to be processed. 200 mL is sufficient for processing 1 plate.
2	Transfer to a 250-mL bottle and invert to mix. Do not store unused 2.0 Wash Buffer. Make 2.0 Wash Buffer fresh daily.

IMPORTANT Precautions

- Avoid touching the bottom of the Filter Plate with your fingers or benchtop at all times.
- Avoid splashing and cross-contamination during all wash steps.
- Do not exceed vacuum pressure of 1–2 inches of Hg during filtration or Capture Bead loss might occur.
- Check Filter Plate for chips and cracks in the bottom. Do not use a cracked plate.
- Do not allow Filter Plates to air dry following washes. Turn off vacuum as soon as filtration is complete and immediately add the next solution.
- Following the last wash in each series, thoroughly blot the top and bottom of the Filter Plate to completely remove all of the Wash Buffer.
- Do not apply pressure to the tops of Filter Plate wells during manipulations or the contents of the wells might be forced through the filter resulting in reagent loss.
- Minimize the exposure of beads to room light.

Hybridizing the 2.0 Pre-Amplifier

To hybridize the 2.0 Pre-Amplifier:

Step	Action
1	Prepare 2.0 Pre-Amplifier Working Reagent: a. Centrifuge the 2.0 Pre-Amplifier briefly to collect the contents at the bottom of the tube. b. Add 36 µL of 2.0 Pre-Amplifier to 12 mL of Amplifier Diluent. c. Invert several times to mix. **IMPORTANT** The Amplifier Diluent is a viscous solution. Pipet carefully to ensure that the entire contents are expelled from the pipette and that the working reagent is completely transferred to the reagent reservoir.
2	Pre-wet the Filter Plate: a. Fill a 100 mL-capacity reagent reservoir with 2.0 Wash Buffer. b. Seal unused wells with a foil seal. c. Place the Filter Plate on the Filter Plate Holder. d. Add 100 µL of 2.0 Wash Buffer to each well of the Filter Plate and incubate at room temperature for 1 minute. e. Transfer the Filter Plate to the vacuum manifold and filter the 2.0 Wash Buffer. **IMPORTANT** Ensure vacuum pressure does not exceed 1–2 inches of Hg, and filtration rate is 5–15 seconds. Adjust pressure if necessary. f. Turn off vacuum and return the Filter Plate to the Filter Plate Holder. **Note** See "Appendix V: Automated Filter Plate Washing Method (optional)" on page 39 for an alternative automated filter plate washing method.
3	**IMPORTANT** This step should be completed within 10 minutes. Transfer the overnight hybridization mixture to the Filter Plate and filter: a. Remove the Hybridization Plate from the shaking incubator, and centrifuge at 240 x g for one minute. **IMPORTANT** Adjust temperature of shaking incubator to **50 °C ± 1 °C**. Verify temperature using a QuantiGene Incubator Temperature Validation Kit. b. Pipet up and down 5 times, then completely transfer the hybridization mixture to the Filter Plate. c. Transfer the Filter Plate to the vacuum manifold and filter. d. Immediately proceed to the next step.
4	Wash away the unbound sample: a. Carefully add 200 µL of 2.0 Wash Buffer to each of the wells, filter completely, then turn vacuum off. b. Repeat step 4a two more times for a total of three washes. c. Blot the top and bottom of the Filter Plate to completely remove all traces of 2.0 Wash Buffer and return to the Filter Plate Holder. d. Immediately proceed to the next step.

To hybridize the 2.0 Pre-Amplifier: *(continued)*

Step	Action
5	Start the 2.0 Pre-Amplifier hybridization: a. Transfer 2.0 Pre-Amplifier Working Reagent to a 25 mL-capacity reagent reservoir. b. Add 100 µL of 2.0 Pre-Amplifier Working Reagent to each assay well. c. **Very gently**, seal the Filter Plate with a foil Plate Seal, then blot the bottom of the Filter Plate on a clean, dry paper towel. d. Place the Filter Plate on the inverted Plate Lid, and incubate for 1 hour in the Vortemp shaking incubator at **50 °C ± 1 °C** and 600 rpm.

Hybridizing the 2.0 Amplifier

To hybridize the 2.0 Amplifier:

Step	Action
1	Prepare 2.0 Amplifier Working Reagent: a. Centrifuge the 2.0 Amplifier briefly to collect the contents at the bottom of the tube. b. Add 36 µL of 2.0 Amplifier to 12 mL of Amplifier Diluent. c. Invert several times to mix. **IMPORTANT** The Amplifier Diluent is a viscous solution. Pipet carefully to ensure that the entire contents are expelled from the pipette and that the working reagent is completely transferred to the reagent reservoir.
2	Wash away the unbound 2.0 Pre-Amplifier: a. Remove the Filter Plate from the shaking incubator. b. Remove the foil Plate Seal from the assay wells and filter completely, then turn off vacuum. c. Carefully add 200 µL of 2.0 Wash Buffer to each of the wells, filter completely, then turn vacuum off. d. Repeat step 2c for a total of two washes. e. Blot the top and bottom of the Filter Plate to completely remove all traces of 2.0 Wash Buffer and return to the Filter Plate Holder. f. Immediately proceed to the next step.
3	Start the 2.0 Amplifier hybridization: a. Transfer 2.0 Amplifier Working Reagent to a 25 mL-capacity reagent reservoir. b. Add 100 µL of 2.0 Amplifier Working Reagent to each assay well. c. **Very gently**, seal the Filter Plate with a foil Plate Seal, then blot the bottom of the Filter Plate on a clean, dry paper towel. d. Place the Filter Plate on the inverted Plate Lid, and incubate for 1 hour in the Vortemp shaking incubator at **50 °C ± 1 °C** and 600 rpm.

Hybridizing the Label Probe

To hybridize the Label Probe:

Step	Action
1	Prepare the Label Probe Working Reagent: a. Centrifuge Label Probe briefly to collect the contents at the bottom of the tube. b. Add 36 µL of Label Probe to 12 mL of Label Probe Diluent. c. Vortex for 15 seconds to mix.
2	Wash away the unbound 2.0 Amplifier: a. Remove the Filter Plate from the shaking incubator. b. Remove the foil Plate Seal from the assay wells and filter completely. c. Carefully add 200 µL of 2.0 Wash Buffer to each assay well, filter completely, then turn off the vacuum. d. Repeat step 2c for a total of two washes, blot the top and bottom of the Filter Plate, and return to the Filter Plate Holder. e. Immediately proceed to the next step.

To hybridize the Label Probe: *(continued)*

Step	Action
3	Hybridize the Label Probe: a. Transfer the Label Probe Working Reagent to a 25 mL-capacity reagent reservoir. b. Add 100 µL of the Label Probe Working Reagent to each assay well. c. **Very gently** reseal the Filter Plate with a foil Plate Seal, then blot the bottom of the Filter Plate on a clean, dry paper towel. d. Place the Filter Plate on the inverted Plate Lid, and incubate for 1 hour in the shaking incubator at **50 °C ± 1 °C** and 600 rpm.

Binding the SAPE

To bind the SAPE:

Step	Action
1	Prepare the SAPE Working Reagent: a. Briefly vortex SAPE to mix, then briefly centrifuge to collect the contents at the bottom of the tube. b. Add 36 µL of SAPE to 12 mL of SAPE Diluent. c. Vortex for 15 seconds to mix, and protect from light.
2	Wash away unbound Label Probe: a. Remove the Filter Plate from the shaking incubator. b. Remove the foil Plate Seal from the assay wells, filter completely, then turn off the vacuum. c. Carefully add 200 µL of 2.0 Wash Buffer to each assay well, filter completely, then turn off the vacuum. d. Repeat step 2c for a total of two washes, blot the top and bottom of the Filter Plate, and return to the Filter Plate Holder. e. Immediately proceed to the next step.
3	Bind the SAPE: a. Transfer the SAPE Working Reagent to a 25 mL-capacity reagent reservoir. b. Add 100 µL of the SAPE Working Reagent to each assay well. c. **Very gently** reseal the Filter Plate with a foil Plate Seal, then blot the bottom of the Filter Plate on a clean, dry paper towel. d. Place the sealed Filter Plate on the Filter Plate Holder and completely wrap the assembly (sealed Filter Plate and Filter Plate Holder) with aluminum foil. e. Place on a shaking platform at **room temperature** and 600 rpm for **30 minutes**.

Detecting the Signal

To detect the signal:

Step	Action
1	Wash away the unbound SAPE: a. Fill a 100 mL-capacity reagent reservoir with **SAPE Wash Buffer**. b. Remove the Filter Plate from the shaking platform. c. Remove the aluminum foil and Plate Seal from the assay wells, filter completely, then turn off the vacuum. d. Carefully add 200 µL of SAPE Wash Buffer to each well, filter completely, then turn off the vacuum. e. Repeat step 1d for a total of two washes, blot the top and bottom of the Filter Plate, and return it to the Filter Plate Holder. f. Immediately proceed to the next step.
2	Prepare the plate for analysis on a Luminex instrument: a. Add 130 µL of **SAPE Wash Buffer** to each assay well. b. **Very gently** reseal the Filter Plate with a foil Plate Seal, then blot the bottom of the Filter Plate on a clean, dry paper towel. c. Place the sealed Filter Plate on the Filter Plate Holder, then wrap the assembly (sealed Filter Plate and Filter Plate Holder) in aluminum foil. **Note** At this point, the plate can be stored at room temperature in the dark for up to 2 hours or at 4 °C for 24 hours (without shaking). Proceed to the next step when you are ready to read the plate. d. Place on a shaking platform at **room temperature** and 600 rpm for 2–5 minutes, then read immediately. **IMPORTANT** If running more than 1 plate at a time, leave the 2nd plate at room temperature in the dark (without shaking). Once the 1st plate has been read and the instrument wash protocol has been completed, place the 2nd plate on a shaker platform at room temperature shaking at 600 rpm for 2–5 minutes, then read immediately. **IMPORTANT** Refer to “Luminex Setup and Operation” on page 14 for instrument setup and operation recommendations.

Section III: Troubleshooting

Low Assay Signal or Poor Sensitivity

Troubleshooting low assay signal or poor sensitivity:

Probable Cause	Recommended Actions
Number of RNA transcripts below of detection limit	Increase the sample input. If using 2.0 Capture Beads (polystyrene), try using the high sensitivity setting for Luminex instruments. This may result in a little more sensitivity at the low end but will impact the high end signals as instrument saturation occurs at 20,000 MFI.
Signal amplification reagent is incorrectly prepared	Carefully add the correct amounts of 2.0 Pre-Amplifier, 2.0 Amplifier, Label Probe and SAPE to the appropriate Diluent and mix thoroughly.
Expired reagents were used	Reagents are good for 6 months from date of receipt.
Sub-optimal assay conditions	Follow the recommended incubation times and temperature. Shake the Filter Plate during all incubations. Verify the temperature of incubations throughout the procedure. If you are not using the recommended Vortemp shaker, see “Appendix IV: Validating Alternative Shaking Incubators” on page 37 for validating alternative shakers.
Photobleaching of SAPE	Protect SAPE from light throughout the procedure.
Incorrect wash buffer was used	Use SAPE Wash Buffer to wash away unbound SAPE.
Instrument needle is partially clogged	Replace or clean needle according to the manufacturer’s recommendations.
Incomplete cell lysis	Refer to the appropriate sample processing kit product inserts for detailed procedures and troubleshooting.
Significant RNA degradation	Refer to the appropriate sample processing kit product inserts for detailed procedures and troubleshooting.

High Background Signal

Troubleshooting high background signal:

Probable Cause	Recommended Actions
Probe Set was not heated prior to adding to the assay wells	Heat the Probe Set to 95 °C for 5 minutes, then place on ice until use.
Sub-optimal assay conditions	Follow the recommended incubation times and temperature. Shake the Filter Plate during all incubations. Verify the temperature of incubations throughout the procedure. If you are not using the recommended Vortemp shaker, see “Appendix IV: Validating Alternative Shaking Incubators” on page 37 for validating alternative shakers.
Hybridization mixture was left at room temperature for too long prior to overnight hybridization	Minimize the setup time and ensure that the hybridization mixture/sample is not left at room temperature for more than 10 minutes.
Expired reagents were used	Reagents are good for 6 months from the date of receipt.

Low Assay Precision (High CV)

Troubleshooting low assay precision:

Probable Cause	Recommended Actions
Inaccurate pipetting	♦ Use only calibrated, precision pipettes ♦ Affix tips securely ♦ Use a new tip for each transfer ♦ Pipet slowly and carefully, avoiding bubbles
Residual Wash Buffer	Filter Wash Buffer completely and blot the top and bottom of the Filter Plate with absorbent paper towels to remove residual Wash Buffer after the last wash in each series.
Non-homogenous samples	Warm samples to 37 °C to dissolve any precipitate, and vortex briefly before use. If samples contain particulates, centrifuge at high speed for 15 minutes, then transfer supernatants to a new tube and repeat centrifugation and transfer step before use.
Incomplete cell lysis	Refer to the appropriate sample processing kit product inserts for detailed procedures and troubleshooting.
Instrument needle is partially clogged	Replace or clean the needle according to the manufacturer's recommendations.
Bubble introduction into Luminex fluidics	Check Luminex probe for proper height, then run instrument debubbling protocol. Make sure every well contains 130 µL of SAPE Wash Buffer and verify the Luminex sample size is set to 100 µL.
Using buffers containing precipitates	Eliminate precipitates by warming to 37 °C for 30 minutes followed by gentle swirling.

Low Bead Count

Troubleshooting low bead count:

Probable Cause	Recommended Actions
Capture Beads settled or clumped in stock tube	Vortex Capture Beads for 30 seconds immediately prior to adding to Working Plex Set.
Capture Beads were not resuspended prior to transfer to the Filter Plate	Pipet up and down to resuspend the Capture Beads in the Hybridization Plate prior to transfer of the hybridization mixture to the Filter Plate.
Vacuum setting too high	Do not exceed a vacuum of 1–2 inches of Hg.
Vacuum on for too long when removing the buffer from the wells	Do not apply vacuum for longer than 15 seconds after the buffer is completely drained from each well of the Filter Plate.
Filter Plate not shaken enough prior to reading	Shake the Filter Plate at 600 rpm for at least two minutes to resuspend the beads before reading the plate.
Incorrect Luminex probe height	Adjust the height of the probe following the procedures supplied with your Luminex system.
Instrument needle is partially clogged	Replace or clean needle according to the manufacturer's recommendations.
Luminex or BioPlex system is clogged	Refer to the troubleshooting guide provided with the system.

Troubleshooting low bead count: *(continued)*

Probable Cause	Recommended Actions
Bubble introduction into Luminex fluidics	Check Luminex probe for proper height, then run instrument debubbling protocol. Make sure every well contains 130 µL of SAPE Wash Buffer and verify the Luminex sample size is set to 100 µL.

Poor Assay Linearity

Troubleshooting poor assay linearity:

Probable Cause	Recommended Actions
Inadequate sample preparation	Refer to the appropriate sample processing kit product inserts for detailed procedures and troubleshooting.
Instrument saturation	Signals >20,000 MFI on Luminex instruments are saturated.
Assay saturation	When using 2.0 Capture Beads, assay saturation occurs when RNA transcripts exceed approximately 5,000,000. When using 2.0 Mag Capture Beads, assay saturation occurs when RNA transcripts exceed approximately 1,500,000. Perform serial dilution of sample to ensure appropriate fold change is observed. See “Determining Assay Linearity” on page 12 for more information.

Clogged Filter Plate Wells

Troubleshooting clogged filter plate wells:

Probable Cause	Recommended Actions
Inadequate sample preparation	Refer to the appropriate sample processing kit product inserts for detailed procedures and troubleshooting.

Section IV: Appendices

Appendix I: Setting Up Luminex Instrument for QuantiGene Plex 2.0 Assays

Instrument Settings

Calibrate the instrument using the standard PMT setting (may be referred to as low by some suppliers). The key information in this table is related to the bead calibration required and setting of the DD gate based on the type (Standard or Magnetic) of Capture Beads being used. Because of continual software updates, we recommend that you contact your Luminex instrument supplier and verify the latest recommendations for setup of Standard and Magnetic xMAP/Luminex beads.

When beads are injected into the flow cell, a small percentage can clump and go through the flow cell as doublets. The DD gate of Doublet Discriminator gate allows for discrimination of doublet formation. When initially setting the DD gate, follow the recommendations in the table below. In some cases, you might need to adjust the DD gate around the largest peak which represents the singlet beads. Adjustments can be made during the processing of the first sample.

IMPORTANT Use SAPE Wash Buffer. The bead position on the DD gate might change if the incorrect buffer is used to suspend the Capture Beads during reading.

Software / Instrument Platform and Supplier	2.0 Capture Bead (polystyrene Luminex xMAP)		2.0 Mag Capture Bead (magnetic Luminex MagPlex)		Sample Size (µL)	Timeout (sec)	Bead Events/Bead region
	Bead Calibration	DD gate	Bead Calibration	DD gate			
xPonent/Luminex v3.0	**Standard** PROTOCOL> BEAD TYPE, select Standard	Automatically set	**Magnetic**[a] PROTOCOL> BEAD TYPE, select magnetic	Automatically set	100	40	100
IS100/Luminex v2.3	**Standard** NEW BATCH> CREATE ASSAY TEMPLATE>Acq. Detail, enter DD gate value	4,300–15,000	**Standard** NEW BATCH> CREATE ASSAY TEMPLATE>Acq. Detail, enter DD gate value	5,000–25,000	100	40	100
MasterPlex CT/MiraiBio v1.0	**Standard** ACQUISITION SET-UP>SET-UP enter DD gate value	4,300–10,000	**Standard** ACQUISITION SET-UP>SET-UP, enter DD gate value	5,000–25,000	100	40	100
MasterPlex CT/MiraiBio v1.2	**Standard** ACQUISITION TEMPLATE SET-UP>BEAD TYPE,select Standard	Automatically set	**Magnetic**[a] ACQUISITION TEMPLATE SET-UP>BEAD TYPE,select Magnetic	Automatically set	100	40	100
Bio-Plex 4.0/BioRad	**Standard** RUN PROTOCOL> ADVANCE WINDOW SETTING, enter DD gate values	4,300–10,000	**Standard** RUN PROTOCOL> ADVANCE WINDOW SETTING, enter DD gate values	5,000–25,000	100	45	100
Bio-Plex 5.0/BioRad	**Standard** SELECT ANALYTE> EDIT PANEL, select xMAP	Automatically set	**Standard** SELECT ANALYTE> EDIT PANEL, select MagFlex	Automatically set	100	45	100

Software / Instrument Platform and Supplier	2.0 Capture Bead (polystyrene Luminex xMAP)		2.0 Mag Capture Bead (magnetic Luminex MagPlex)		Sample Size (µL)	Timeout (sec)	Bead Events/Bead region
	Bead Calibration	DD gate	Bead Calibration	DD gate			
Applied Station/Applied Cytometry	**Standard** TEMPLATE SET-UP> enter DD gate value	4,300–10,000	**Standard** TEMPLATE SET-UP> enter DD gate value	5,000–25,000	100	40	100

a. You must use MagPex calibrator beads for calibration (MagPlex Calibration Beads, Luminex P/N MCAL1-05 and MagPlex Control Beads, P/N MCON1-05).

Appendix II: Recommended Maintenance for Luminex Instruments

About Luminex Maintenance

For optimal results, we strongly recommend that you clean the sample probe/needle on a regular basis according to the manufacturer's recommendations. Probe/needles that have rusty or salt deposits should be replaced (Luminex P/N CN-0007-01).

After Each Run

Clean the probe/needle after each run as indicated in the following table.

Step	Number of Times	Solution
Sanitize	2x	20% bleach
Backflush	3x	none
Alcohol wash	3x	70% ethanol
Wash	4x	Distilled water

Before Shut Down

Clean the probe/needle before shutting down the instrument as indicated in the following table.

Step	Number of Times	Solution
Sanitize	2x	20% bleach
Backflush	3x	none
Alcohol wash	3x	70% ethanol
Wash	4x	Distilled water
Soak	1x	Water

Appendix III: Common Housekeeping Genes and their Relative Expression Levels

The table below lists the common housekeeping genes and general guidelines for the linear working range based on the number of cells/well. These guidelines assume the Luminex instrument has been setup and calibrated as instructed in this user guide.

We recommend including a minimum of 3 housekeeping genes, each at a different relative expression level, in all Plex Sets. For example, include housekeeping genes representing medium high, medium, and low relative expression levels.

Housekeeping Genes and Expression Levels		**Guidelines for cells/well**	
Relative Expression Level	**Common Housekeeping Gene**	**2.0 Capture Beads**	**2.0 Mag Capture Beads**
High	ACTB, B2M, GAPDH, PPIA, RPL19, RPL23, RPL32, RPLR0, RPS3, RPS18, RPS20	200–10,000	50–5,000
Medium high	LDHA, PGK1, PPIB, RPL13A, UBC	400–80,000	100–30,000
Medium	HPRT, POLR2A, TFRC	1,600–80,000	400–60,000
Low	ATP6V1A, GUSB, HMBS, TBP, TNX2	8,000–80,000	2,000–80,000

Appendix IV: Validating Alternative Shaking Incubators

About Alternative Incubators

Alternative equipment must meet the minimum specifications stated in “Required Reagents and Equipment” on page 8.

Systems Tested by Panomics

We have validated 2 systems using this procedure. Both show 2-fold lower sensitivity than the Vortemp incubator.

- WVR FM1350 incubator oven and Labnet, orbit P4 microplate shaker
- ATRBiotech Multitron II Micro shaker incubator (www.artbiotech.com)

Validating Procedure

Before You Start

This procedure requires 3 Panomics thermometers (QuantiGene Incubator Temperature Validation Kit, P/N QS0517).

Calibrating the Oven

To calibrate the oven:

Step	Action
1	Place 3 Panomics thermometers into the oven, spreading them evenly throughout the plate platform.
2	Set the oven to the recommended assay temperature.
3	After 4 hours, record the readings of the 3 thermometers.
4	Adjust the oven temperature, raise the temperature if readings are lower, lower the temperature if the readings are higher.
5	Wait another 2 hours and read the thermometers. Adjust, if necessary.
6	Repeat steps 4–5 until the oven reaches the recommended assay temperature.
7	Note the oven setting.
8	Repeat this procedure for all temperature settings used in the assay.

Verifying the Optimal Temperature

To verify the optimal temperature:

Step	Action
1	Perform a temperature titration of your assay to ensure the temperature setup is optimal. We recommend performing a +/- 2 °C titration from the recommended assay temperature. An example experiment is shown below.

To verify the optimal temperature: *(continued)*

Step	Action
2	Analyze the results for the highest signal, lowest background, and lowest limit of detection.

Sample Volume (µL)	53 °C	55 °C	57 °C
80			
40			
20			
10			
5			
0-background			

Verifying Uniformity

To verify the uniformity:

Step	Action
1	Load the same amount of sample across the entire plate.
2	Run the assay and compare the well-to-well variation. The average CV across all wells should be less than 15%.

Appendix V: Automated Filter Plate Washing Method (optional)

About Automated Washing

Here we provide guidelines for setting up and running the BioTek ELx50/8FMW Microplate Strip Washer.

IMPORTANT Use of this automated washing system requires additional purchase of QuantiGene Plex 2.0 Wash Buffer and SAPE Wash Buffer. Each use requires a 40 mL priming volume.

IMPORTANT Review the BioTek ELx50 General Usage Guidelines before setting up and running the QuantiGene assay.

Creating a Program

To create a program for QuantiGene 2.0 assay in a 96-well Filter Plate:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Create Prime Program called New_Buffer_Prime and enter the following settings.
♦ Prime Volume = 40 mL
♦ Prime Flow Rate = 6 (600 µL/well/sec)
♦ Soak after Prime = No</td></tr>
<tr><td>2</td><td>Create individual Dispense Programs D1 and D2.
(Main menu >DEFINE > CREATE > DISP > Select DISP program > Enter program name (D1)...)
<table>
<tr><th>Parameter</th><th>D1</th><th>D2</th></tr>
<tr><td>Dispense Volume</td><td>200 µL</td><td>130 µL</td></tr>
<tr><td>Dispense Flow Rate</td><td>5 (500 µL/well/sec)</td><td>5 (500 µL/well/sec)</td></tr>
<tr><td>Dispense Height</td><td>120 (15.240 mm)</td><td>120 (15.240 mm)</td></tr>
<tr><td>Horizontal Dispense Position</td><td>0 (0 mm)</td><td>0 (0 mm)</td></tr>
<tr><td>Prime Before Start?</td><td>No</td><td>No</td></tr>
</table></td></tr>
<tr><td>3</td><td>Create 6 individual Aspiration Programs, A1–A3 (for cells and RNA) and A4–A6 (for tissue, blood, and FFPE).
(Main Menu > DEFINE > Create > ASPIR > Select ASPIR program > Enter program name (A1)...)
<table>
<tr><th colspan="4">Cell Lysates and Total RNA Sample Types</th></tr>
<tr><th>Parameter</th><th>A1</th><th>A2</th><th>A3</th></tr>
<tr><td>Aspirate Type</td><td>Bottom (BOT)</td><td>Bottom (BOT)</td><td>Bottom (BOT)</td></tr>
<tr><td>Aspirate Time</td><td>8 sec</td><td>12 sec</td><td>15 sec</td></tr>
</table>
<table>
<tr><th colspan="4">Tissue, Blood, and FFPE Sample Types</th></tr>
<tr><th>Parameter</th><th>A4</th><th>A5</th><th>A6</th></tr>
<tr><td>Aspirate Type</td><td>Bottom (BOT)</td><td>Bottom (BOT)</td><td>Bottom (BOT)</td></tr>
<tr><td>Aspirate Time</td><td>13 sec</td><td>17 sec</td><td>20 sec</td></tr>
</table></td></tr>
</table>

To create a program for QuantiGene 2.0 assay in a 96-well Filter Plate: *(continued)*

Step	Action
4	Create Link programs for day 2 processing. (Main Menu > DEFINE > CREATE > MORE > LINK > Enter NAME (1st WASH) > Select LINK 01 > ENTER ASPIR (A1) > ENTER DISP (D1) > ENTER ASPIR (A1) > ENTER DISP (D1) > ENTER ASPIR (A1) > ENTER DISP (D1) > ENTER ASPIR (A2)...)

Cell Lysates and Total RNA Sample Types

First Wash Link 01 (wash overnight reaction)	**Second Wash Link 02 (wash out unbound PreAmp and Amp)**	**Third Wash Link 03 (wash out unbound Label Probe)**	**Fourth Wash Link 04 (wash out unbound SAPE)**
Aspirate: 8 sec (A1)	Aspirate: 12 sec (A2)	Aspirate: 8 sec (A1)	Aspirate: 8 sec (A1)
Dispense: 200 µL (D1)	Dispense: 200 µL (D1)	Dispense: 200 µL (D1)	Dispense: 200 µL (D1)
Aspirate: 8 sec (A1)	Aspirate: 8 sec (A1)	Aspirate: 8 sec (A1)	Aspirate: 8 sec (A1)
Dispense: 200 µL (D1)	Dispense: 200 µL (D1)	Dispense: 200 µL (D1)	Dispense: 200 µL (D1)
Aspirate: 8 sec (A1)	Aspirate: 15 sec (A3)	Aspirate: 15 sec (A3)	Aspirate: 15 sec (A3)
Dispense: 200 µL (D1)			Dispense: 130 µL (D2)
Aspirate: 12 sec (A2)			

Tissue, Blood, and FFPE Sample Types

First Wash_1 Link 05 (wash overnight reaction)	**Second Wash_1 Link 06 (wash out unbound PreAmp and Amp)**	**Third Wash_1 Link 07 (wash out unbound Label Probe)**	**Fourth Wash_1 Link 08 (wash out unbound SAPE)**
Aspirate: 13 sec (A4)	Aspirate: 17 sec (A5)	Aspirate: 13 sec (A4)	Aspirate: 13 sec (A4)
Dispense: 200 µL (D1)	Dispense: 200 µL (D1)	Dispense: 200 µL (D1)	Dispense: 200 µL (D1)
Aspirate: 13 sec (A4)	Aspirate: 13 sec (A4)	Aspirate: 13 sec (A4)	Aspirate: 13 sec (A4)
Dispense: 200 µL (D1)	Dispense: 200 µL (D1)	Dispense: 200 µL (D1)	Dispense: 200 µL (D1)
Aspirate: 13 sec (A4)	Aspirate: 20 sec (A6)	Aspirate: 20 sec (A6)	Aspirate: 20 sec (A6)
Dispense: 200 µL (D1)			Dispense: 130 µL (D2)
Aspirate: 17 sec (A5)			

Procedure for Cell and RNA Sample Types

To wash cell and RNA samples:

Step	Action
1	Verify the washer is set at LOW vacuum level and leave the vent port on the carrier open.
2	Place QuantiGene 2.0 Wash Buffer in the appropriate bottle.
3	Run the Prime program, New_Buffer_Prime.
4	Run Link program 01, First Wash (overnight hybridization reaction). (Main Menu > RUN > MORE > LINK > LINK: SELECT LINK 01 (FIRST WASH) > ENTER NUMBER OF STRIP > PLACE PLATE IN CARRIER AND PRESS <START>)

To wash cell and RNA samples: *(continued)*

Step	Action
5	Run Link program 02, Second Wash (remove unbound Pre-Amplifier).
6	Run Link program 02, Second Wash (remove unbound Amplifier).
7	Run Link program 03, Third Wash (remove unbound Label Probe).
8	Place SAPE Wash Buffer in the appropriate bottle.
9	Run the Prime program, New_Buffer_Prime.
10	Run Link Program 04, Fourth Wash (replace QuantiGene 2.0 Wash Buffer with SAPE Wash Buffer).
11	PRESS<STOP>TO QUIT.

Procedure for Tissue, Blood and FFPE Sample Types

To wash tissue, blood, and FFPE samples:

Step	Action
1	Verify the washer is set at LOW vacuum level and leave the vent port on the carrier open.
2	Place QuantiGene 2.0 Wash Buffer in the appropriate bottle.
3	Run the Prime program, New_Buffer_Prime.
4	Run Link program 05, First Wash_1 (overnight hybridization reaction). (Main Menu > RUN > MORE > LINK > LINK: SELECT LINK 05 (FIRST WASH) > ENTER NUMBER OF STRIP > PLACE PLATE IN CARRIER AND PRESS <START>)
5	Run Link program 06, Second Wash_1 (remove unbound Pre-Amplifier).
6	Run Link program 06, Second Wash_1 (remove unbound Amplifier).
7	Run Link program 07, Third Wash_1 (remove unbound Label Probe).
8	Place SAPE Wash Buffer in the appropriate bottle.
9	Run the Prime program, New_Buffer_Prime.
10	Run Link Program 08, Fourth Wash_1 (replace QuantiGene 2.0 Wash Buffer with SAPE Wash Buffer).
11	PRESS<STOP>TO QUIT.

Appendix VI: Blank Plate Maps